ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 33: 207–211 (2020)
DOI 10.3897/herpetozoa.33.e60214

First record of *Rhacophorus verrucopus* Huang, 1983 from Myanmar

Shuo Liu[1], Ye Htet Lwin[2], Ruichang Quan[2,3], Song Li[1]

1 *Kunming Natural History Museum of Zoology, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, China*
2 *Southeast Asia Biodiversity Research Institute, Chinese Academy of Sciences, Yezin, Nay Pyi Taw 05282, Myanmar*
3 *Center for Integrative Conservation, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla, Yunnan 666303, China*

http://zoobank.org/6A7AD27F-91CA-4E9F-AA9C-0B5AB864021B

Corresponding author: Song Li (lis@mail.kiz.ac.cn)

Academic editor: G.Gollmann ♦ **Received** 31 October 2020 ♦ **Accepted** 19 November 2020 ♦ **Published** 1 December 2020

Abstract

We report the first country record of *Rhacophorus verrucopus* Huang, 1983 from Myanmar, based on one specimen collected from Htamanthi Wildlife Sanctuary, Sagaing Division. Morphologically, the specimen shows good agreement with the original description of *R. verrucopus* and phylogenetically, it is clustered with the specimen of *R. verrucopus* from Medog, Tibet, China with strong support. This is also the first record of *R. verrucopus* from outside of China.

Key Words

16S rRNA, Htamanthi Wildlife Sanctuary, new record, tree frog

Introduction

Rhacophorus Kuhl & van Hasselt, 1822 occurs in the tropical and temperate zones of East, South and Southeast Asia and, recently, was partitioned into three genera including *Rhacophorus*, *Leptomantis* Peters, 1867 and *Zhangixalus* Li, Jiang, Ren & Jiang, 2019 (in Jiang et al. 2019). Now the genus *Rhacophorus* includes 44 recognised species with a distribution range from India, Bangladesh, Bhutan, Myanmar, Thailand, Laos, Cambodia, Vietnam, Malaysia, Indonesia and Philippines, as well as extreme southern and south-western China (mainly in Hainan, Guangxi, Yunnan and Tibetan) (Jiang et al. 2019; Frost 2020). *Rhacophorus verrucopus*, a species which was described and named from Medog, Tibet, China, was previously known only from the type locality (Huang 1983; AmphibiaChina 2020; Frost 2020).

Myanmar is an important component of the Indo-Burma biodiversity hot-spot and its northern region lies at a biogeographic crossroads where the faunas of China, Indochina, India and Himalaya converge (Wogan et al. 2008). In recent years, the researchers of Southeast Asia Biodiversity Research Institute, Chinese Academy of Science have found many new species and new records of animals and plants in northern Myanmar (Li and Quan 2017). During our field survey in northern Myanmar in 2019, a specimen of *Rhacophorus* with a small and elongated body and distinct tarsal projections was collected. Molecular comparison indicated this individual to be *R. verrucopus*. Herein, we describe this new record for Myanmar in detail.

Materials and methods

Field surveys were conducted in Htamanthi Wildlife Sanctuary, Sagaing Division, Myanmar. The specimen was collected and euthanised with ethyl acetate and then fixed in 75% ethanol for storage after taking photographs. Liver tissue sample was preserved in 99% ethanol for molecular analysis. The specimen was deposited in Southeast Asia Biodiversity Research Institute, Chinese Academy of Sciences (abbreviation: SEABRI; address: Yezin, Nay Pyi Taw, Myanmar).

Copyright *Shuo Liu et al.* This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Total genomic DNA was extracted from liver tissue. Tissue sample was digested using proteinase K and subsequently purified, following standard phenol/chloroform isolation and ethanol precipitation. A fragment of encoding partial 16S rRNA gene was amplified using primer pairs 16Sar/16Sbr (Palumbi et al. 1991). Polymerase chain reaction (PCR) amplifications were performed in 50 µl reactions using the following cycling conditions: initial denaturing step at 95 °C for 4 min; 35 cycles of denaturing at 94 °C for 60 s, annealing at 51 °C for 60 s and extending at 72 °C for 60 s; and a final extension step of 72 °C for 10 min. Sequencing was conducted directly using the corresponding PCR primers. The new sequence was deposited in GenBank under accession number MW275978. Outgroups were selected according to Jiang et al. (2019) and Nguyen et al. (2020). Homologous and outgroup sequences were obtained from GenBank (Table 1).

Sequences were aligned using ClustalW with default parameters in MEGA 7 (Kumar et al. 2016). Uncorrected pairwise distances between species were calculated in MEGA 7 with the parameters Transitions + Transversions, Uniform rates and Pairwise deletion. The best substitution model GTR+G was the Bayesian Information Criterion (BIC) in jModelTest 2.1.7 (Darriba et al. 2012). Bayesian Inferences were performed in MRBAYES v3.2.6 (Ronquist et al. 2012). Two runs were performed simultaneously with four Markov chains starting from the random tree. The chains were run for 1 000 000 generations and sampled every 100 generations. The first 25% of the sampled trees were discarded as burn-in after the standard deviation of split frequencies of the two runs was less than 0.01. The remaining trees were then used to create a consensus tree and to estimate Bayesian posterior probabilities (BPPs). Maximum Likelihood analysis was performed in Raxml-GUI 1.5 (Silvestro and Michalak 2012) and nodal support values were estimated by 1,000 rapid bootstrap replicates.

Measurements were taken with a digital caliper to the nearest 0.1 mm. Morphological terminology followed Fei et al. (2009). Measurements included: snout-vent length (SVL, from tip of snout to vent); head length (HL, from tip of snout to rear of jaw); head width (HW, width of head at widest point); snout length (SL, from tip of snout to anterior border of eye); internarial distance (IND, distance between nares); interorbital distance (IOD, minimum distance between upper eyelids); eye diameter (ED, diameter of exposed portion of eyeball); tympanum diameter (TD, greater of tympanum vertical and horizontal diameters); distance from nostril to eye (DNE, from nostril to anterior border of eye); forearm and hand length (FHL, from elbow to tip of third finger); tibia length (TL, distance from knee to heel); and foot length (FL, from proximal end of inner metatarsal tubercle to tip of fourth toe).

Results

The obtained sequence alignment for the 16S gene was 510 bp long. SEABRI2019120056 clustered with *R. verrucopus* from Medog, Tibet, China with strong support (Fig. 1). The genetic distance between

Table 1. Species used for molecular phylogenetic analysis.

Species	Voucher	Locality	Accession No.
Ingroup			
Rhacophorus annamensis	VNMN 4092	Gia Lai, Kon Ka Kinh, Vietnam	LC010568
	VNMN 4090	Dak Nong, Nam Nung, Vietnam	LC010566
Rhacophorus bipunctatus	CAS235303	Bee Hoe, Chin, Myanmar	JX219444
	CAS229913	Putao, Kachin, Myanmar	JX219445
Rhacophorus calcaneus	VNMN 4093	Dak Lac, Chu Yang Sin, Vietnam	LC010573
	KIZ 528	Bi Doup, Lam Dong, Vietnam	JX219450
Rhacophorus exechopygus	VNMN 4107	Gia Lai, Kon Ka Kinh, Vietnam	LC010585
	VNMN 4108	Gia Lai, Kon Ka Kinh, Vietnam	LC010586
Rhacophorus helenae	AMS R 173230	Binh Thuan, Vietnam	JQ288087
	UNS 00450	Dong Nai, Vietnam	JQ288088
Rhacophorus hoabinhensis	IEBR A.2016.18	Hoa Binh, Vietnam	LC331096
	VNMN A.2016.16	Hoa Binh, Vietnam	LC331097
Rhacophorus kio	VNMN 4110	Gia Lai, Kon Ka Kinh, Vietnam	LC010589
	VNMN 4111	Ha Giang, Bac Quang, Vietnam	LC010590
Rhacophorus nigropalmatus	Rao081203	Malaysia	JX219438
	Rao081204	Malaysia	JX219437
Rhacophorus orlovi	VNMN 3067	Ha Tinh, Huong Son, Vietnam	LC010598
	VNMN 4115	Nghe An, Pu Huong, Vietnam	LC010600
Rhacophorus rhodopus	SCUM 060692L	Mengyang, Yunnan, China	EU215531
	X219440	Lvchun, Yunnan, China	JX219440
Rhacophorus robertingeri	VNMN 4123	Gia Lai, Kon Ka Kinh, Vietnam	LC010613
	VNMN 3446	Kon Tum, Kon Plong, Vietnam	LC010615
Rhacophorus spelaeus	IEBR A.2011.1	Khammouan, Lao	LC331095
Rhacophorus translineatus	Rao6237	Medog, Tibet, China	JX219449
Rhacophorus verrucopus	6254 Rao	Medog, Tibet, China	JX219436
	SEABRI2019120056	Htamanthi, Sagaing, Myanmar	MW275978
Outgroup			
Zhangixalus duboisi	VNMN 4102	Sa Pa, Lao Cai, Vietnam	LC010580
Zhangixalus dugritei	SCUM 051001L	Baoxing, Sichuan, China	EU215541
Leptomantis gauni	FMNH273928	Sarawak, Bintulu, Malaysia	JX219456
Leptomantis penanorum	ZRC 1.12116	Sarawak, Bintulu, Malaysia	JN377350
Buergeria buergeri	IABHU 41011	Hiroshima, Japan	AB127977

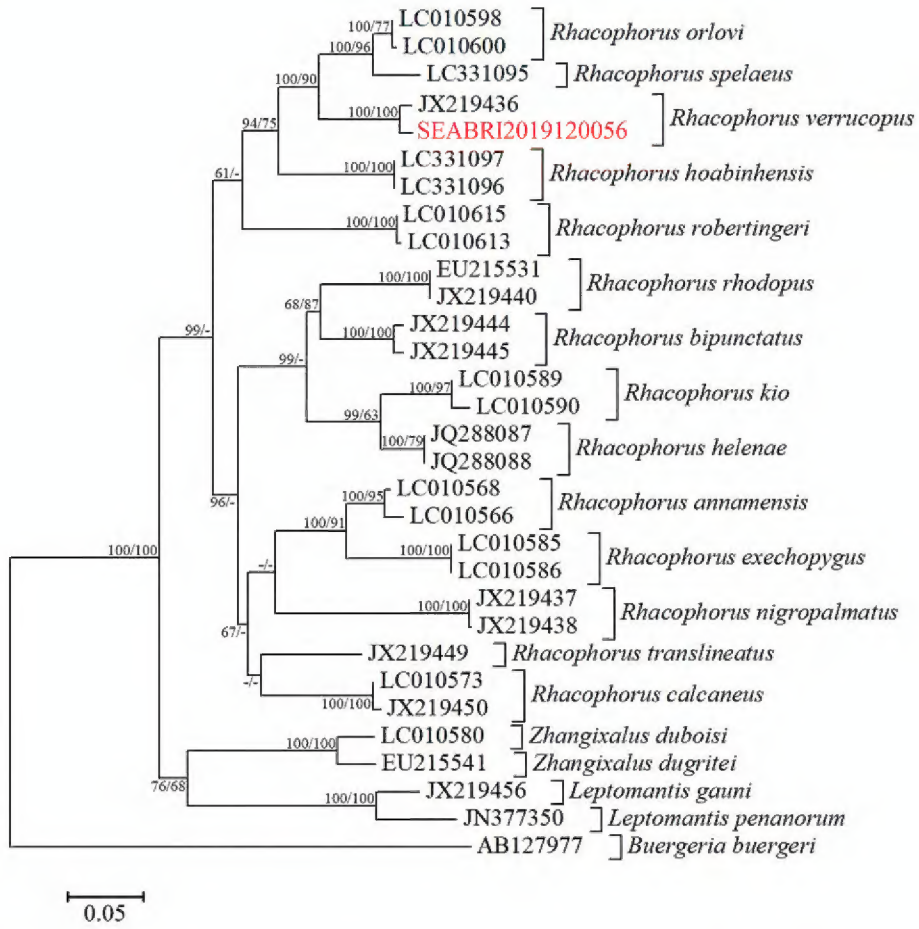

Figure 1. Maximum Likelihood tree of partial *Rhacophorus* species inferred from 16S rRNA gene sequences (Numbers before slashes indicate Bayesian posterior probabilities and numbers after slashes indicate bootstrap support for Maximum Likelihood analyses. The symbol "–" represents values below 60). The specimen collected from Myanmar is indicated by red.

SEABRI2019120056 and *R. verrucopus* from Medog, Tibet, China was 1.2% (Table 2). Therefore, we considered SEABRI2019120056 belongs to *R. verrucopus*.

Taxonomic account

Rhacophorus verrucopus Huang, 1983

Fig. 2

Specimen examined. Adult female (SEABRI2019120056) collected on 21 December 2019 by the local guides from Htamanthi Wildlife Sanctuary, Sagaing Division, Myanmar (25°21'59"N, 95°22'59"E, 90 m elevation).

Morphological description. Morphological characters of the specimen from Myanmar agreed well with the original description of Huang (1983) and subsequent descriptions of Fei et al. (2009, 2012). Body small and elongate, SVL 52.0 mm; head length (HL 17.6 mm) larger than width (HW 15.7 mm); snout slightly sharp, canthus rostralis distinct, loreal region slightly oblique; nostril at tip of snout, internarial distance (IND 4.3 mm) narrower than interorbital distance (IOD 9.3 mm); tympanum (TD 3.1 mm) rounded, slightly larger than half eye diameter (ED 5.4 mm), separated from eye by 1.6 mm; pupil transverse, eye diameter larger than half snout length (SL 7.7 mm). Vomerine teeth present; tongue attached anteriorly, deeply notched posteriorly.

Forearm and hand length (FHL 17.8 mm) no more than half SVL; relative length of fingers I < II < IV < III; tips of all fingers expanded into discs with transverse grooves, disc of first finger small, disc of third finger largest, its width nearly equal to tympanum; webbing formula I 1-1 II 0-1 III 1-1/2 IV; subarticular tubercles distinct, formula 1, 1, 2, 2; rows of tubercles present on palms; inner metacarpal tubercle large and flat, outer metacarpal tubercle absent.

Hind limbs long, tibia (TL 24.2 mm) about half SVL and longer than thigh (22.9 mm) and foot (FL 20.4 mm); relative length of toes I < II < III < V < IV; tips of toes expanded into discs, smaller than those of fingers; webbing formula I 0-0^{-} II 0-1^{+} III 0-1 IV 1-0 V, subarticular tubercles distinct, formula 1, 1, 2, 3, 2; rows of tubercles present on pes; inner metatarsal tubercle flat, outer metatarsal tubercles absent.

Skin of dorsum smooth; supratympanic fold thin and distinct; outer edge of forearm with light coloured granules arranged in serrated shape; dermal calcars present on heels forming tarsal projections; granules above vent forming transverse skin fold; ventral skin covered with small flat granules.

Colour in life. Colour pattern similar to the holotype of *R. verrucopus*. Dorsal surface greyish-yellow with scattered small brownish-black spots; limbs with very indistinct transverse stripes; each side of thigh and inner sides of shank, tarsus and foot orangish-red; webbing between fingers orangish-yellow, webbing between toes orangish-red; ventral surface greyish-white, belly sides light yellow; pupil black, iris greyish-yellow.

Table 2. Divergence (P-distance; %) between and within homologous species and *Rhacophorus verrucopus* estimated from 16S gene sequences.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 *Rhacophorus annamensis*														
2 *Rhacophorus bipunctatus*	9.6													
3 *Rhacophorus calcaneus*	10.4	10.8												
4 *Rhacophorus exechopygus*	6.9	9.5	10.1											
5 *Rhacophorus helenae*	10.5	7.2	10.7	12.3										
6 *Rhacophorus hoabinhensis*	9.1	13.4	10.4	13.5	9.6									
7 *Rhacophorus kio*	10.8	9.0	12.2	11.9	5.8	12.3								
8 *Rhacophorus nigropalmatus*	9.9	13.4	12.0	10.5	10.5	14.1	13.3							
9 *Rhacophorus orlovi*	10.9	11.5	10.8	13.2	12.2	8.7	12.0	11.8						
10 *Rhacophorus rhodopus*	10.9	9.1	11.6	11.3	9.5	14.4	11.1	13.5	12.4					
11 *Rhacophorus robertingeri*	11.0	11.0	9.9	13.3	11.7	10.3	11.3	12.2	10.6	11.9				
12 *Rhacophorus spelaeus*	11.8	11.5	10.7	13.5	12.0	9.2	12.4	12.6	3.6	12.4	9.9			
13 *Rhacophorus translineatus*	9.0	10.8	9.2	9.5	10.6	12.2	12.1	12.1	10.8	11.5	10.5	11.0		
14 *Rhacophorus verrucopus* (China)	12.2	13.5	11.3	13.7	9.9	10.6	10.7	14.1	7.5	13.9	10.5	9.0	12.7	
15 *Rhacophorus verrucopus* (Myanmar)	12.3	9.8	10.5	13.7	9.5	10.7	11.2	11.1	7.8	12.4	11.0	9.2	11.0	1.2

Figure 2. The specimen (SEABRI2019120056) collected from Myanmar in life. **A.** Dorsal view; **B.** ventral view; **C.** lose-up view of anterior part of thigh; **D.** close-up view of posterior part of thigh; **E.** close-up view of hand; **F** close-up view of foot.

Ecological notes. The specimen was found at night on a bush approximately 0.8 m above the ground near a large river. Several eggs were visible through the skin of the belly. The eggs were yellow and large. The breeding period of this species remains unknown.

Discussion

Morphologically, the specimen of *R. verrucopus* from Myanmar shows good agreement with the original description, except for minor differences in colouration. In the original description, the colour of the upper eyelids was greyish-brown and there were greyish-brown transverse bands present on limbs. However, the upper eyelids of the specimen from Myanmar were the same colour as the dorsal surface and the transverse bands on the limbs were nearly invisible.

Rhacophorus verrucopus was known previously only from Tibet, China. This is the first record of *R. verrucopus* from Myanmar and from outside of China (Fig. 3). According to the original description, this species inhabited the area between 850 m and 1500 m elevation in the type locality (Huang 1983). The new location in Myan-

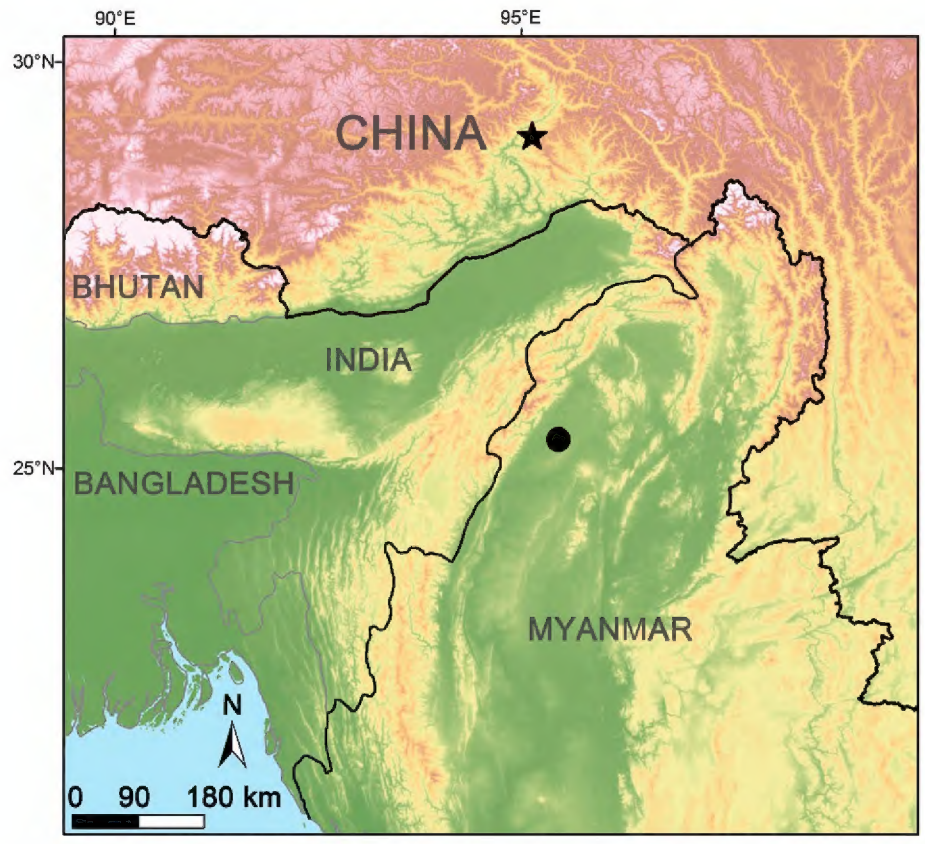

Figure 3. Collection site of *Rhacophorus verrucopus* (black dot) in Myanmar and the type locality (black star) in Tibet, China.

mar is approximately 440 km away from the type locality in China and the altitude (90 m elevation) of the new location is much lower than the type locality.

Acknowledgements

The field survey in Myanmar was undertaken at the invitation of the Republic of the Union of Myanmar, Ministry of Natural Resources and Environmental Conservation, Forest Department, Forest Research Institute. We thank them for the invitation. Thanks also to the staff and the local guides of Myanmar for their help in the field.

References

AmphibiaChina (2020) The database of Chinese amphibians. Electronic Database. http://www.amphibiachina.org [Accessed on 20 Oct 2020]

Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. Nature Methods 9: 772–772. https://doi.org/10.1038/nmeth.2109

Fei L, Hu SQ, Ye CY, Huang YZ (2009) Fauna Sinica (Vol. 2). Amphibia Anura. Science Press, Beijing, 957 pp.

Fei L, Ye CY, Jiang JP (2012) Colored Atlas of Chinese Amphibians and Their Distributions. Sichuan Publishing House of Science and Technology, Chengdu, 620 pp.

Frost DR (2020) Amphibian Species of the World: an Online Reference. Version 6.1. Electronic Database. https://amphibiansoftheworld.amnh.org/index.php [Accessed on 20 October 2020]

Huang YZ (1983) A new species of flying frog from Xizang – *Rhacophorus verrucopus*. Acta Herpetologica Sinica 2(4): 63–65.

Jiang DC, Jiang K, Ren JL, Wu J, Li JT (2019) Resurrection of the genus *Leptomantis*, with description of a new genus to the family Rhacophoridae (Amphibia: Anura). Asian Herpetological Research 10: 1–12. https://doi.org/10.16373/j.cnki.ahr.180058

Kuhl H, Hasselt JC van (1822) Uittreksels uit breieven van de Heeren Kuhl en van Hasselt, aan de Heeren C. J. Temminck, Th. van Swinderen en W. de Haan. Algemeene Konst-en Letter-Bode 7: 99–104.

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33(7): 1870–1874. https://doi.org/10.1093/molbev/msw054

Li SQ, Quan RC (2017) Taxonomy is the cornerstone of biodiversity conservation – SEABRI reports on biological surveys in Southeast Asia. Zoological Research 38(5): 213–214. https://doi.org/10.24272/j.issn.2095-8137.2017.061

Nguyen TT, Ninh HT, Orlov N, Nguyen TQ, Ziegler T (2020) A new species of the genus *Zhangixalus* (Amphibia: Rhacophoridae) from Vietnam. Journal of Natural History 54: 257–273. https://doi.org/10.1080/00222933.2020.1754484

Palumbi SR, Martin A, Romano S, McMillan W, Stice L, Grabowski G (1991) The Simple Fool's Guide to PCR. University of Hawaii Press, Honolulu, 94 pp.

Peters WCH (1867) Herpetologische Notizen. Monatsberichte der königlichen Akademie der Wissenschaften zu Berlin 1867: 13–37.

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61: 539–542. https://doi.org/10.1093/sysbio/sys029

Silvestro D, Michalak I (2012) raxmlGUI: a graphical front-end for RAxML. Organisms Diversity and Evolution 12(4): 335–337. https://doi.org/10.1007/s13127-011-0056-0

Wogan GOU, Vindum JV, Wilkinson JA, Koo MS, Slowinski JB, Win H, Thin T, Kyi S, Oo S, Lwin K, Shein A (2008) New country records and range extensions for Myanmar Amphibians and Reptiles. Hamadryad 33(1): 83–96.